EFFECTS OF CSK HOMOLOGOUS KINASE OVEREXPRESSION ON HER/NEU-MEDIATED SIGNAL TRANSDUCTION PATHWAYS IN BREAST CANCER CELLS

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Random onset of breast cancers has in many cases been correlated with increased HER2/Neu (also termed ErbB-2) expression and Src family tyrosine kinases activity. Association of c-Src with these receptor tyrosine kinases (PTKs) is an integral part of the signaling events mediated by the receptors, and may contribute to the malignant transformation of cells. Increased Src kinase activity observed in HER2/Neu-induced tumors results from the ability of the Src-SH2 domain to directly interact with HER2/Neu in a tyrosine phosphorylation-dependent manner. Since HER2/Neu and pp60src play a role in breast cancer and are altered during malignant transformation and tumor progression, it is important to characterize the regulation of these protein kinase activities, and the likely interactions of these kinases with each other.

Src family kinase activity is inhibited by the phosphorylation of a conserved, carboxy-terminal tyrosine. The protein tyrosine kinase responsible for this phosphorylation is Csk. We and others identified a second member of the Csk family – Csk Homologous Kinase (CHK). CHK has been suggested to have a specific role in breast cancer and the potential to be a target of breast cancer drug development. CHK, which is specifically expressed in primary breast cancer specimens, but not in normal breast tissues, phosphorylates Src and down-regulates its activity. Previous biochemical data also suggested that CHK acts as a negative growth regulator of human breast cancer. Furthermore, the interaction between the CHK-SH2 domain and pTyr1248 of the HER2/Neu receptor is specific and critical for CHK function. 1.

We hypothesize that: (i) CHK is able to antagonize the growth-promoting signals that are mediated by HER2/Neu and Src kinases; (ii) enhancement of the binding affinity of CHK to the HER2/Neu protein might further increase the antitumor effects of CHK in breast cancer cells. Therefore, we aimed: (i) to investigate the effects of CHK on downstream signaling from the HER2/Neu receptor; and (ii) to assess the possibility of enhancing the inhibitory effects of CHK on HER2/Neu-mediated signaling.

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NEGATIVE REGULATION OF ERBB-2 AND THE ROLE OF CBL IN MAMMARY TUMORIGENESIS

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Upon activation of the ErbB-2/Neu receptor tyrosine kinase, tyrosine residues in the cytoplasmic tail may be phosphorylated and become docking sites for phosphotyrosine interacting proteins. Five such tyrosine autophosphorylation sites in the cytoplasmic tail of ErbB-2 have been previously characterized (Dankort et al. MCB 17, 1997). In vitro transformation assays suggests that four of the five tyrosine sites (Y1144, Y1201, 1226/7, Y1253) mediate positive signaling pathways whereas Y1028 acts as a negative regulator of ErbB-2 activity. To determine the physiological significance of this transformation data, we employed a targeted erbB-2 knock-in strategy. Expression of the endogenous erbB-2 gene was replaced with expression of an erbB-2 cDNA encoding a tyrosine to phenylalanine mutation at either Y1028, Y1144 (Grb2 binding site), or Y1226/7 (Shc binding site). Homozygous animals expressing the knock-in mutant erbB-2 cDNA were healthy and fertile. We are currently evaluating the effects of these mutations on mammary gland development. Interestingly, we also observed that the Y1028F mutation resulted in a significant increase in the level of ErbB-2 protein expression. These observations are consistent with the negative regulatory effect of Y1028. Although the mechanism for the negative regulation on ErbB-2 activity is currently unknown, one candidate protein is the proto-oncogene c-Cbl. We demonstrate an in vitro association of c-Cbl with ErbB-2 but this was not dependent on phosphorylation of Y1028. Instead, we observed that c-Cbl associates specifically with phosphorylated Y1144 and to a lesser extent with Y1226/7. However, c-Cbl association with ErbB-2 did not correspond with receptor ubiquitination and downregulation. c-Cbl is a multi-adaptor protein and also mediates positive signals. We are currently investigating the role of c-Cbl in mammary gland development and tumorigenesis. By identifying the mechanisms for the downregulation of receptor tyrosine kinases involved in breast cancer, this may facilitate an understanding of how some breast cancers progress and become resistant to treatment and may lead to the development or refinement of therapeutic targets.

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INVOLVEMENT OF TRUNCATED PROTEIN TYROSINE PHOSPHATASE 1B IN INSULIN SIGNALING AND APOPTOSIS IN BREAST CANCER

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The balance of tyrosine phosphorylation is tightly controlled by expression and activation of tyrosine protein kinases and phosphatases (PTP) and lose of this control can result in multiple diseases including cancer and diabetes. One phosphatase that may play a role in both of these diseases is protein tyrosine phosphatase 1B (PTP1B). Previous studies suggested that PTP1B dephosphorylates multiple substrates and its overexpression can suppress insulin and other growth factor signaling events. Mouse PTP1B knockout studies confirmed a negative regulatory role for this protein in insulin signaling and some studies have suggested that it may provide a novel target for type II diabetes. However, the role of this phosphatase in insulin signaling in breast and other cancers has not been thoroughly examined. Interestingly, it is known that woman from ethnic groups with a high incidence of type II diabetes have a correspondingly low incidence of breast cancer, suggesting that common regulators of insulin signaling may have opposing effects on these diseases. To determine if PTP1B plays a role in this process, a truncated and cytoplasmic form of PTP1B (tPTP1B) was cloned and expressed in both insulin/IGF-1 responsive and unresponsive breast cancer cells. The truncated form of PTP1B was constructed to mimic the activated and cytoplasmic form of this protein found in cells treated with calcium ionophores, cytokines or other cellular stresses. Wild-type, phosphatase-dead, and substrate-trapping variants of tPTP1B were cloned into FLAG-tagged and tet-repressive vectors to investigate its cellular distribution, potential substrates and its effects on insulin signaling and apoptosis. Transient expression of wild-type tPTP1B resulted in a reduction in cell viability and tet-regulated expression blunted insulin and IGF-1 signaling and growth in responsive breast cancer cells. The effects were associated with altered tyrosine phosphoryaltion and multiple substrates previously shown to be targets of PTP1B (IR, IRS-1, EGFr, c-src, Stat 5, p130Cas) are being examined. Together, these results suggest that a cytoplasmic form of PTP1B may mediate changes in growth and survival of breast cancer cells.

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INSULIN-LIKE GROWTH FACTOR-I REGULATES ESTROGEN RECEPTOR-ALPHA TRANSCRIPTIONAL ACTIVITY BY MULTIPLE PATHWAYS IN BREAST CANCER

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Estrogen and insulin-like growth factor-I (IGF-I) both induce breast cancer cell proliferation. While estrogen acts through a nuclear hormone receptor to stimulate cell growth, IGF-I acts through a transmembrane tyrosine kinase receptor to affect growth. IGF-I activates transcriptional activity of estrogen receptor-alpha (ER-alpha) in the absence of steroid hormone, although the mechanism is not fully understood. To determine which signaling pathways are involved in IGF-I-induced activation of the estrogen receptor, we treated the estrogen receptor-positive human breast cancer cell line MCF7 stably transfected with an estrogen-response element luciferase reporter construct with inhibitors of downstream signaling pathways. Treatment with IGF-I (5nM) or estrogen (1nM) resulted in a 2-3 fold increase in luciferase activity. Cotreatment with IGF-I and estrogen resulted in enhanced luciferase activity than compared to either treatment alone. Previous studies have shown that mitogen-activated extracellular regulated protein kinase (ERK) and phosphatidylinositol-3-kinase (PI3K) mediate many of the biological effects of IGF-I. Inhibition of ERK1/2 by U0126 (20μM) or treatment with the PI3K inhibitor LY294002 (25μM) blunted the IGF-I-induced increase in luciferase activity. U0126 but not LY294002 also blocked the estrogen-induced increase in luciferase activity. Interestingly, the p38 inhibitor SB203580 (20 μM) also suppressed IGF-I mediated activation of luciferase activity. However, treatment of MCF7 cells with IGF-I (5nM) did not increase the level of phosphorylation of p38, as determined by phosphospecific immunoblot analysis. These studies suggest that multiple IGF-I-activated signaling pathways contribute to transactivation of ER-alpha. In contrast, estrogen-induced activation of ER-alpha does not appear to require the same set of signaling pathways. While ERK signaling appears to be central to estradiol and IGF-I action, additional IGF-I-activated signaling pathways play a role in regulating ER-alpha function.

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INSULIN RECEPTOR SUBSTRATE 1 IS A RATE-LIMITING FACTOR IN ANAPLASTIC LYMPHOMA KINASE RECEPTOR SIGNALING

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Anaplastic lymphoma kinase (ALK) is a transmembrane receptor tyrosine kinase (RTK) with an apparent molecular mass of 220 kDa. It was originally discovered in a chromosomal translocation associated with anaplastic large cell lymphomas. In this translocation, the ALK tyrosine kinase domain was fused to nucleophosmin (NPM) resulting in a t(2,5) translocation, and generated a constitutively active ALK kinase. Since the original discovery, full length ALK and several of other ALK fusion oncogenes have been detected and linked to other diseases such as anaplastic large cell lymphomas (ALCL) and neuroblastoma. In our lab, we found that the mRNA for ALK is expressed in 8 of the 18 breast cancer cell lines. Interestingly, these eight breast cancer cell lines have lost their sensitivity to estradiol (ER-) and have been established from invasive types of tumors. Recently, our lab reported that pleiotrophin (PTN) is a ligand for ALK and that insulin receptor substrate 1 (IRS-1) is a major substrate of ALK (Stoica et al. 2001, JBC, 276:16772-9). Furthermore, we observed that midkine (MK), PTN's only other known family member, can also bind and activate ALK. PTN and MK are secreted heparin binding growth factors that are implicated in various biological activities such as neurite outgrowth, mitogenesis and angiogenesis. Our findings suggested that ALK could be involved in the activity of PTN or MK as a mitogenic and angiogenic factor. In the present study, we used a cell line that is devoid of ALK and IRS family members to investigate the importance of IRS-1 as a substrate for ALK signaling.

32D cells are murine myeloid progenitor cells that are interleukin-3 (IL-3) dependent. Abrogation of IL-3 dependence in these cells has been shown to suggest a transformation potential. These cells also do not proliferate in response to PTN or MK. Stable 32D transfectants were generated expressing human ALK alone (32D/ALK), human IRS-1 alone (32D/IRS-1), or ALK and IRS-1 together (32D/ALK/IRS-1). Here we report that only 32D cells co-expressing ALK and IRS-1 can abrogate the absolute requirement of IL-3 in these cells. Moreover, only 32D/ALK/IRS-1 cells show activated downstream signaling molecules such as MAP kinase and PI3-kinase, which are known to be involved in mitogenic and anti-apototic pathways. These findings are further validated by experiments with ALK ATP binding mutant (ALK-ΔATP) and ALK IRS-1 binding mutant (ALK-ΔIRS-1). Proliferation assays and apoptosis assays show that cells expressing IRS-1 and ALK mutants lose their ability to survive in the absence of IL-3. The ability of 32D/ALK/IRS-1 cells to survive without IL-3 might be due to endogenous expression of midkine in these cells. This endogenous expression of MK can activate ALK, which activates IRS-1 and other downstream molecules, creating a constitutively activated autocrine loop. In conclusion, this study suggests that IRS-1 is a rate-limiting factor in the survival of pathway utilized by the ALK receptor.

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A HUMANIZED SINGLE-CHAIN ANTIBODY AGAINST THE TYPE I IGF RECEPTOR RENDERS BREAST CANCER CELLS REFRACTORY TO THE MITOGENIC EFFECTS OF INSULIN-LIKE GROWTH FACTOR-I

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Insulin-like growth factors (IGFs) stimulate proliferation of breast cancer cells via the type I IGF receptor (IGF-IR). In MCF-7 cells, we have shown that IGF-I treatment results in phosphorylation of the adaptor protein insulin receptor substrate-1 (IRS-1). IRS-1 then recruits other signaling molecules resulting in the activation of downstream mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. Thus, inhibition of IGF-IR activation should inhibit IGF action in breast cancer cells. We have previously shown that a humanized single chain antibody against IGF-IR (IGF-IR scFv-Fc) stimulated IGF-IR signaling in vitro when MCF-7 cells were treated with scFv for 10 minutes. It also stimulated the proliferation of MCF-7 cells in monolayer growth assays. Moreover, scFv did not inhibit IGF-I's ability to activate IGF-IR or stimulate MCF-7 cell growth. In contrast, scFv has been shown to partially inhibit xenograft growth of MCF-7 cells in mice. In this study, we examined the mechanism by which scFv could inhibit tumor growth in mice. When MCF-7 cells were treated with 250 nM scFv for different time periods, scFv downregulated IGF-IR levels after 2h and the levels were greatly reduced after 24h. In contrast, treatment with IGF-I over the same time period did not affect IGF-IR levels. To determine if scFv made MCF-7 cells refractory to further IGF-I effects, MCF-7 cells were pretreated with scFv for 24h to downregulate IGF-IR and then stimulated with IGF-I. 24h pretreatment of cells with scFv inhibited the ability of IGF-I to phosphorylate IRS-1 and blocked subsequent MAPK and PI3K activation. In contrast, cells treated with IGF-I for 24h still retained the ability to activate downstream signaling pathways in response to IGF-I. Moreover, pretreatment of MCF-7 cells with scFv rendered them refractory to further proliferation by IGF-I and inhibited anchorage independent growth. The mechanism by which scFv inhibits tumor growth does not appear to involve antibody-dependent cell-mediated cytotoxicity (ADCC) as it did not enhance lysis of MCF-7 cells in an in vitro cytotoxicity assay. Our data suggest that downregulation of type I IGF-IR by an antibody could be a potential anti-IGF therapy in breast cancer.

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THE TYROSINE KINASE JAK2 AND ITS POTENTIAL ROLE IN BREAST CANCER

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The development and progression of breast cancer is mediated by a complex sequence of genetic alterations and concomitant cellular responses. Over the past few years, a considerable amount of data has been published suggesting that the Stat transcription factors are involved in breast cancer. For example a recent study has demonstrated that overexpression of epidermal growth factor receptor (EGFR), which is frequently observed in breast cancer, activates Stat5b. Furthermore, constitutive phosphorylation of Stat3 is associated with breast cancer. Despite these observations the upstream kinase, or kinases, responsible for the abnormal and persistent activation of the Stat factors have received less attention. Since both Stat3 and Stat5 can be activated by the upstream tyrosine kinase Jak2, we have investigated the role of Jak2 in breast cancer development and progression. To study the potenital contribution of Jak2 to cancer development we first examined its role in normal development. Utilizing Jak2-null embryos and an embryonic mammary gland transplantation technique we have demonstrated that Jak2 is absolutely required for pregnancy-mediated development of mammary epithelium. Thus in the absence of functional Jak2 cell proliferation was reduced by 95 % . Immunohistochemical analyses using markers indiciative of distinct stages of mammary gland development revealed that Jak2-null glands retained ductal features and failed to acquire markers indicative of alveolar cells, suggestive of an arrest in functional development. We are currently generating a conditinal knockout out of the Jak2 gene to more precisely determine the effect of Jak2 deletion on mammary epithelial development. To establish the role of Jak2 in breast cancer we are examining the phosphorylation status of Jak2, and the downstream target Stat5, in tumors isolated from a Brca1/p53 mouse model. Tumors develop in the Brca1/p53 mice within 7 - 8 months and the pathalogical features of these tumors are considered to be very similar to that observed in humans making this an ideal model to study. As an additional approach we are utilizing a multiple cancer tissue array to determine if constitutive activation of Jak2 and Stat5 are associated with primary human breast tumors.

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GROWTH FACTOR REGULATION OF THE ETK TYROSINE KINASE IN BREAST CANCER CELLS

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The mechanisms by which growth factor induced intracellular signaling pathways mediate breast cancer cell adhesion and migration are still unclear. We have previously demonstrated that treatment of metastatic MDA-MB-435 breast cancer cells with epidermal growth factor or heregulin-beta (HRG-b) can induce rapid increases in adhesion and migration that require the lipid kinase phosphatidylinositol 3-kinase (PI 3-K). Recent studies in lymphoid cells have demonstrated that the Itk tyrosine kinase, a member of the Bruton's tyrosine kinase (Btk) family of tyrosine kinases, regulates integrin-mediated adhesion in a PI 3-K-dependent manner. Since epithelial cells express the Btk tyrosine kinase family member Etk/Bmx, we examined Etk expression, activation and membrane localization in breast cancer cell lines that express the HER2, HER3, and HER4 growth factor receptors. Using Western blotting analysis, we have shown that Etk is expressed in the metastatic breast cancer cell line MDA-MB-435, but not in the non-metastatic MCF-7 and T47D breast cancer cell lines. HRG-b stimulation of MDA-MB-435 cells results in tyrosine phosphorylation of endogenous Etk. HRG-b stimulation also results in tyrosine phosphorylation of wild-type Etk, but not dominant-negative or kinase-inactive Etk, expressed transiently in MCF-7 cells. We have also utilized confocal microscopy to examine the intracellular localization of GFP-Etk fusion proteins in unstimulated and HRG-b stimulated MCF-7 cells. While wild-type GFP-Etk is localized primarily to the cytosol in unstimulated MCF-7 cells, HRG-b stimulation results in rapid membrane localization of GFP-Etk. HRG-b-mediated membrane localization of GFP-Etk requires the pleckstrin homology (PH) domain of Etk, as a GFP-Etk construct lacking the PH domain (GFP-deltaPH Etk) does not localize to the membrane following HRG-b stimulation. These results demonstrate that HRG-b stimulation results in activation of Etk and membrane recruitment of Etk that requires the Etk PH domain. Since our prior studies demonstrated that inhibition of Etk expression blocks integrin-mediated migration of 435 cells, these studies also suggest that Etk may participate in growth factor-mediated regulation of breast cancer cell adhesion and migration.

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THE BREAST TUMOR KINASE BRK IS A NUCLEAR TYROSINE KINASE THAT PHOSPHORYLATES AND REGULATES THE RNA-BINDING PROTEIN SAM68 AND THE SAM68-LIKE MAMMALIAN PROTEINS SLM-1 AND SLM-2

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The breast tumor kinase BRK is an epithelial specific intracellular tyrosine kinase that is distantly related to the Src family of tyrosine kinases and has a similar structure, but it lacks a myristoylation signal. It is expressed in normal differentiating cells of the skin and the gastrointestinal tract but no BRK expression has been detected in the normal mammary gland. However BRK is expressed in a high percentage of human breast tumors and breast tumor cell lines.

We found that BRK is a nuclear tyrosine kinase that phosphorylates the RNA binding protein Sam68 (Src associated during mitosis, 68 kDa). BRK interacts with Sam68 through its SH3 and SH2 domains, and the proline rich P3 region of Sam68 is required for BRK SH3 binding. Endogenous BRK and Sam68 colocalize in Sam68/SLM nuclear bodies (SNBs) in a number of human breast tumor cell lines. In functional studies, the expression of BRK abolished the ability of Sam68 to bind RNA and act as a cellular Rev homologue.

In addition to Sam68, we have found that, BRK also phosphorylates the Sam68-like mammalian proteins SLM-1 and SLM-2. We examined expression of Sam68, SLM-1, and SLM-2 during development of the mouse mammary gland using RNase protection assays and in situ hybridization. In the normal mouse mammary gland only expression of Sam68 was detected. We are currently examining expression of SLM-1 and SLM-2 in human breast tumor cell lines.

While Sam68 is a substrate for Src family kinases during mitosis, BRK is the first identified tyrosine kinase that can phosphorylate Sam68 and regulate its activity within the nucleus, where it resides during most of the cell cycle. Since Sam68 has been implicated in cell cycle regulation, increased expression of BRK may contribute to the development of breast cancer by altering the ability of Sam68 to regulate cell growth.

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MONITORING TYROSINE KINASE RNA EXPRESSION WITH cDNA MICROARRAYS

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Aberrant expression of receptor or cytosolic tyrosine kinase (tk) genes and, in particular their hyper-expression, are common phenomena in breast cancer. Knowledge about the expression levels of all tk genes in a cell might contribute significantly to an understanding of the processes of tumor development and progression. Based on our experiences cloning tk genes, we developed a PCR-based method that determines the expression profile of up to 100 tyrosine kinase genes in a very few cells. This method relies on degenerate primers designed to amplify gene-specific sequences between two conserved domains within a tk transcript. The primer binding sites on the various transcripts are approximately the same distance apart so the PCR products can be size-selected and purified to greatly improve probe specificity. The amplified sequences between priming sites are sufficiently divergent to determine an expression profile by cDNA microarray hybridization. Since our method relies on well-understood PCR techniques, it can be extended to single cell analysis.

We summarize here our experiences to date with this innovative methodology. In the course of this and other studies, we cloned cDNAs from approximately 60% of the known family of tk genes. We created our initial arrays with these clones. Our cloning efforts early on suggested the exciting possibility that we might be able to identify 'novel' proteins with tk activity. Using commercially available cDNA clone sources, we will soon add all known tk cDNAs to our arrays. Scanning hardware/software available at Berkeley Lab were used for data collection. Probe synthesis is the most critical step in the expression profiling with cDNA microarrays. Our initial degenerate primers were originally based on the conserved consensus protein sequence of all microarrays. Our most recent primer design significantly reduces the complexity of the degenerate primers by basing their sequence on the compiled list of tk mRNA sequences found in Genebank. Without resorting to extraordinary PCR methods, we are now able to generate probe from the total RNA extracted from as few as 200 cells. We have optimized our wash conditions to generate good signals with low backgrounds. Using a set of phenotypically well-characterized breast cancer cells lines, this system has delivered reproducible data regarding changes in tk gene expression during cell transformation and progression towards a more malignant phenotype.

We are currently refining this method to make a representative tyrosine kinase profile of a single cell and define a minimum set of tk genes that changes expression in concert with tumor progression. Since our method relies on making degenerate primers to sequences that define a gene family, we believe that this approach is generally applicable to profile other gene families such as serine/threonine kinases, transcription factors, and other critical regulators of cellular control that have conserved domains. Such high-precision tools are essential to design individualized treatments for breast cancer patients.

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ANGIOPOIETIN FBG DOMAINS MEDIATE TIE2 BINDING AND DETERMINE LIGAND ACTIVITY

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The endothelial cell-restricted receptor tyrosine kinase (RTK), Tie2, which plays an essential role in developmental angiogenesis, has been linked to progression of breast cancer (Br J Cancer 77, 51-56. 1998.). Agents that block Tie2 slow tumor growth and inhibit metastasis in experimental models of breast cancer (PNAS 95(15), 8829-34. 1998.). Because of its putative role in breast cancer, we have been studying how Tie2 is regulated by a family of structurally related proteins called angiopoietins (Ang). Tie2 is unique among mammalian RTKs in that it has antagonistic ligands, e.g. Ang2, as well as agonistic ligands, e.g. Ang1. In studying the structural basis for the differential activity of Ang1 and Ang2, we previously showed that both Ang1 and Ang2 are comprised of an N-terminal coiled coil domain, which mediates homo-oligomerization, and a C-terminal fibrinogen-like (Fbg) domain that determines ligand activity (JBC 274(42), 30196-201. 1999.). Further characterizing Ang1 and Ang2 interactions with Tie2, we have found that they bind competitively and equally to the Tie2 extracellular domain. Their Fbg domains mediate binding, but dimerization or oligomerization of the Fbg domains is required for binding. Their coiled coil domains normally serve to oligomerize the Fbg domains, but this function can be substituted by other dimerization domains, e.g. the Fc domain of IgG or the c-fos and c-jun dimerization domains. Dimerization of the Ang2 Fbg domain reconstitutes its Tie2 antagonist function, and the Ang2 Fbg domain blocks Tie2 even when multimerized. Interestingly, while dimerized Ang1 Fbg domain binds Tie2, it also blocks Tie2 activity. Higher order oligomerization is required to reconstitute its agonistic function. Combined, these studies suggest that Ang2 antagonizes Ang1 function by competitively binding Tie2. They suggest that Tie2 dimerization is insufficient to induce phosphorylation. They suggest that only the Ang1 Fbg domain can induce the conformational changes required to induce Tie2 phosphorylation, but only when its Fbg domain is multimerized. These results have important implications for the design of tyrosine kinase activators and inhibitors, some of which are now in clinical trials for the treatment of breast cancer.

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